

Adventitious shoot regeneration from leaf explants of southern highbush blueberry cultivars

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Abstract Protocols were developed to optimize adventitious shoot regeneration from four southern highbush blueberry cultivars. Leaf explants from 6 week-old shoots of the four cultivars were excised and cultured on woody plant medium each containing thidiazuron (4.54 or 9.08 µM), zeatin (18.2 µM), or zeatin riboside (5.7 or 11.4 µM) either separately or in combination with α -naphthaleneacetic acid at 2.69 µM. Optimum medium for shoot regeneration was genotype-dependent. Efficient regeneration was obtained at frequencies of 88.9% for ‘Jewel’, 87.8% for ‘Emerald’, 53.3% for ‘Jubilee’ and 87.8% for ‘Biloxi’. Leaf explants of newly developed shoots from the cultures having undergone five subcultures had higher regeneration frequencies than those having undergone two subcultures. Regenerated shoots, 80–100% for each cultivar, rooted in 8 weeks after transplantation to soil. The regeneration systems described have potential use in genetic transformation of southern highbush blueberry cultivars.

Keywords Organogenesis · Plant regeneration · *Vaccinium corymbosum*

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Abbreviations

MS	Murashige and Skoog
NAA	α -naphthaleneacetic acid
TDZ	Thidiazuron
WPM	Woody plant medium

Introduction

Vaccinium fruits are considered health-promoting foods because of the relatively high antioxidant and anti-inflammatory capacities (Prior et al. 1998; Ehlenfeldt and Prior 2001; Conner et al. 2002a; b; Zheng and Wang 2003; USDA-ARS 2007). Three *Vaccinium* fruit crops (blueberry, cranberry, and lingonberry) have been domesticated in the twentieth century (Galletta and Ballington 1996; Lyrene et al. 2003; Hancock et al. 2008a). Of these, the highbush blueberry is by far the most important commercial crop. From 1995 to 2007, worldwide blueberry acreage grew, from 23,116 to 58,601 ha (Lehnert 2008).

Vaccinium cultivars are generated exclusively through traditionally controlled hybridization and deliberate selection. Recombinant DNA and transformation techniques have been reported that can supplement and extend conventional breeding methods for cranberry (Serres et al. 1992, 1997; Polashock and Vorsa 2002a; b; Zeldin et al. 2002) and blueberry (Graham et al. 1996; Song and Sink 2004; Song et al. 2008). While transgenic breeding can play an important role in introducing desirable agronomic traits not possible by conventional breeding into commercial *Vaccinium* varieties, it is obvious that reliable, efficient transformation protocols must first be developed (Polashock and Vorsa 2002a; McCown and Zeldin 2005; Rowland and Hammerschlag 2005; Hancock et al. 2008b).

Plant regeneration is a prerequisite step for genetic transformation and is usually influenced by biotic factors including genotype, explant type, and abiotic factors such as culture media and environmental conditions. Although plant cell totipotency theoretically enables any of the cells to retain the ability to regenerate whole new plants through organogenesis or somatic embryogenesis, the regeneration capacity of plant cells usually varies between species, cultivars, and explant types (Ganeshan et al. 2002). Successful plant regeneration has been reported for five *Vaccinium* species, including lowbush blueberry (*V. angustifolium* Ait.) (Nickerson and Hall 1976; Nickerson 1978, 1980; Dweikat and Lyrene 1988), highbush blueberry (*V. corymbosum* L.) (Billings et al. 1988; Callow et al. 1989; Rowland and Ogden 1992, 1993; Hruskoci and Read 1993; Cao and Hammerschlag 2000; Song and Sink 2004), large cranberry (*V. macrocarpon* Ait.) (Serres et al. 1992, 1997; Marcotrigiano et al. 1996; Qu et al. 2000), lingonberry (*V. vitis-idaea* L.) (Debnath and McRae 2002; Debnath 2005; Meiners et al. 2007), and bilberry (*V. myrtillus* L.) (Shibli and Smith 1996). Most of the regeneration occurred via organogenesis.

For southern highbush blueberries, adventitious shoot regeneration using leaf explants has been reported for two hybrid cultivars ‘Legacy’ (73.4% *V. corymbosum*, 1.6% *V. angustifolium*, and 25% *V. darrowi*) and ‘Ozarkblue’ (77.4% *V. corymbosum*, 3.9% *V. angustifolium*, and 11.3% *V. darrowi*) (Song and Sink 2004; Meiners et al. 2007). In addition, transgenic ‘Legacy’ plants with herbicide resistance have been produced and field trials have been carried out (Song et al. 2007, 2008). However, with the wide differences in genetic composition of southern highbush cultivars (Brevis et al. 2008; Ballington 2009), systematic studies are still needed to optimize regeneration systems for individual cultivars. In the present study, we developed efficient regeneration systems for four selected southern highbush cultivars using leaf explants and found that both cultivar source and physiological status of leaf explants affected shoot regeneration. The protocol reported has potential use in optimizing shoot regeneration systems for other *Vaccinium* species.

Materials and methods

Plant materials and culture media

Softwood cuttings from greenhouse-grown plants of four southern highbush cultivars ‘Biloxi’, ‘Emerald’, ‘Jewel’, and ‘Jubilee’ were surface sterilized with 70% (v/v) ethanol for 30 s and 3% sodium hypochlorite containing 0.02% (v/v) Tween 20 for 10 min, followed by three rinses in sterile deionized water. Single-node explants, about 0.5–2 cm in length, were transferred into culture tubes

(150 × 25 mm) each containing 10 ml modified Murashige and Skoog (1962) medium (MS) containing one-half-strength inorganic salts with 0.4 mg l⁻¹ thiamine-HCl, 100 mg l⁻¹ *myo*-inositol, 25 µM 6(γ,γ dimethylallyl)amino-purine (2-isopentenyladenine) (2ip), 2% sucrose, 0.6% Bacto agar, and pH adjusted to 5.2. After 6 weeks. Six to ten newly developed shoots, 2–6 cm in length, were placed horizontally in 40 × 100 mm glass jars containing 30 ml of modified woody plant medium (WPM) (Lloyd and McCown 1980) containing WPM salts and MS vitamins plus 9.12 µM zeatin, 2.35 mM Ca(NO₃)₂·4H₂O, 2% sucrose, and solidified with 0.6% Bacto agar, pH 5.2 (Rowland and Ogden 1992; Song and Sink 2004). They were cultured for an additional 6 weeks. Proliferating shoots were subcultured every 6–8 weeks. Newly developed shoots from the cultures having undergone two and five subcultures, respectively, were used as the sources for leaf explants except where otherwise stated. All the cultures were maintained at 25°C under a 16-h photoperiod of 30 µE m⁻² s⁻¹ from cool white fluorescent tubes.

All media were autoclaved at 121°C for 20 min at 105 kPa. Zeatin, zeatin riboside, or thidiazuron (TDZ) were filter-sterilized through 0.22 µm Millipore filters, and added to medium cooled to 50–60°C.

Adventitious shoot regeneration

The basal regeneration medium was WPM salts, MS vitamins, 2% sucrose, 0.6% Bacto agar, and pH 5.2. Based on our previous report (Song and Sink 2004) and preliminary shoot regeneration experiments, three plant growth regulators (PGRs), TDZ (4.54 or 9.08 µM), zeatin (18.2 µM), and zeatin riboside (5.7 or 11.4 µM), were tested either separately or each in combination with α-naphthaleneacetic acid (NAA) at 2.69 µM resulting in ten regeneration treatments (Table 1).

Leaf explants, about 4–8 mm long and excluding the three youngest leaves near the tip of each shoot, were excised from 6 week-old shoots newly developed from the cultures that had undergone two and five subcultures on modified WPM containing 9.12 µM zeatin, respectively. After removing the petiole and the distal third portion of the blade, the remaining proximal section was the explant used for shoot regeneration.

Freshly prepared explants, ten per petri-dish (100 × 20 mm) and three dishes per treatment, were placed onto 30 ml regeneration medium with the abaxial surfaces in contact with the medium. Petri dishes were incubated in the dark at 25 ± 1°C for 2 weeks before exposure to a 16-h photoperiod of 30 µE m⁻² s⁻¹ from cool white fluorescent tubes. Transfer of the explants to fresh medium was performed every 4 weeks. After

Table 1 Adventitious shoot regeneration from four southern highbush blueberry cultivars on WPM-based regeneration media after 12 weeks of culture

Source of leaf explants	Treatment	Plant growth regulators (μM)	'Biloxi'			'Emerald'			'Jewel'			'Jubilee'		
			TDZ	Zeatin	NAA riboside	Regeneration (%) ^z			Mean no. shoots ^y	Regeneration (%) ^z	Mean no. shoots ^y	Regeneration (%) ^z	Mean no. shoots ^y	Regeneration (%) ^z
						Mean no. shoots ^y	Regeneration (%) ^z	Mean no. shoots ^y						
From the cultures having undergone two subcultures														
T1	4.54		24.4 e		0.34 ef	46.7 c	1.06 de	0.0 c	0.00 b	0.0 f	0.00 b	0.00 f	0.00 f	
T1 N	4.54		2.69	2.2 g	0.02 f	0.0 f	0.00 e	44.4 b	0.98 b	22.2 d	0.58 d	0.00 f	0.00 f	
T2	9.08			14.4 f	0.20 f	31.1 de	0.81 e	0.0 c	0.00 b	0.0 f	0.00 b	0.00 f	0.00 f	
T2 N	9.08		2.69	0.0 g	0.00 f	0.0 f	0.00 e	0.0 c	0.00 b	0.0 f	0.00 b	0.00 f	0.00 f	
ZR2		5.7		26.7 de	0.52 ef	34.4 de	0.52 e	0.0 c	0.00 b	14.4 e	0.20 ef	0.00 f	0.00 f	
ZR2 N		5.7	2.69	0.0 g	0.00 f	0.0 f	0.00 e	0.0 c	0.00 b	0.0 f	0.00 b	0.00 f	0.00 f	
ZR4		11.4		44.4 c	1.06 de	26.7 e	0.54 e	0.0 c	0.00 b	24.4 cd	0.34 e	0.00 f	0.00 f	
ZR4 N		11.4	2.69	0.0 g	0.00 f	0.0 f	0.00 e	0.0 c	0.00 b	0.0 f	0.00 b	0.00 f	0.00 f	
ZT4		18.2		26.7 de	0.67 ef	36.7 d	0.59 e	0.0 c	0.00 b	0.0 f	0.00 b	0.00 f	0.00 f	
ZT4 N		18.2	2.69	0.0 g	0.00 f	0.0 f	0.00 e	0.0 c	0.00 b	0.0 f	0.00 b	0.00 f	0.00 f	
From the cultures having undergone five subcultures														
T1	4.54		75.6 b		2.48 c	75.6 b	8.66 b	0.0 c	0.00 b	0.0 f	0.00 b	0.00 f	0.00 f	
T1 N	4.54		2.69	2.2 g	1.44 d	31.1 de	1.38 de	88.9 a	13.24 a	53.3 a	3.16 a	0.00 f	0.00 f	
T2	9.08			32.2 d	1.00 de	82.2 ab	12.57 a	0.0 c	0.00 b	0.0 f	0.00 b	0.00 f	0.00 f	
T2 N	9.08		2.69	0.0 g	0.00 f	0.0 f	0.00 e	0.0 c	0.00 b	0.0 f	0.00 b	0.00 f	0.00 f	
ZR2		5.7		75.6 b	3.68 b	81.1 ab	3.02 d	0.0 c	0.00 b	27.8 c	1.13 b	0.00 f	0.00 f	
ZR2 N		5.7	2.69	0.0 g	0.00 f	2.2 f	0.02 e	0.0 c	0.00 b	0.0 f	0.00 b	0.00 f	0.00 f	
ZR4		11.4		87.8 a	5.52 a	84.4 a	10.14 b	0.0 c	0.00 b	36.7 b	0.84 c	0.00 f	0.00 f	
ZR4 N		11.4	2.69	0.0 g	0.00 f	0.0 f	0.00 e	0.0 c	0.00 b	0.0 f	0.00 b	0.00 f	0.00 f	
ZT4		18.2		81.1 b	3.16 b	87.8 a	5.30 c	0.0 c	0.00 b	0.0 f	0.00 b	0.00 f	0.00 f	
ZT4 N		18.2	2.69	0.0 g	0.00 f	0.0 f	0.00 e	0.0 c	0.00 b	0.0 f	0.00 b	0.00 f	0.00 f	

Letters next to values in columns indicate significant difference at $P \leq 0.05$ by Duncan's test. $n = 180$ (number of petri dishes)

^y Mean number shoots/explant (9 petri dishes with 10 explants each)

^z Regeneration (%) = Number of explants with at least one shoot/total number of explants $\times 100$ (9 petri dishes with 10 explants each)

12 weeks culture, regeneration frequency (percentage of the leaf explants with at least one shoot) and number of shoots per explant were recorded.

Ten regenerated explants for each cultivar were randomly selected and transferred in 40×100 mm glass jars each containing 30 ml of modified WPM plus 9.12 μM zeatin for continued growth. The cultures were grown at 25°C under a 16-h photoperiod of $30 \mu\text{E m}^{-2} \text{s}^{-1}$ for 8 weeks. Ten elongated shoots, 3–5 cm in length, were then selected for rooting from the optimum regeneration medium for each cultivar.

Rooting

Rooting of regenerated shoots was conducted as described by Song and Sink (2006). Elongated shoots, 3–5 cm in length, were excised and inserted directly in water-soaked sphagnum moss in 6-paks cell trays (Novosel Enterprises, PA, USA). In each experiment, 20 shoots for each cultivar, including ten regenerated shoots and ten micropropagated shoots, were tested for rooting. The cell trays were covered with transparent plastic covers and cultured at 25°C under a 16-h photoperiod of $30 \mu\text{E m}^{-2} \text{s}^{-1}$. After 6 weeks, the plastic covers were progressively opened and removed. Percentage of rooting was recorded after 8 weeks. All plants were transplanted into 4-inch plastic pots containing water soaked sphagnum peat moss (Fafard Peat Moss Co., Ltd. Inkerman, NB, Canada) and Suremix Perlite planting medium (Michigan Grower Products Inc., Galesburg, MI, USA) (v/v = 1:1). They were watered as needed and fertilized weekly using a nutrient solution of 0.2 g/L 21-7-7 acid fertilizer.

Experimental design and data analysis

All experiments were conducted three times in completely randomized designs. Data for shoot regeneration were analyzed for significance by the standard analysis of variance (ANOVA) with mean separation by Duncan's test ($P = 0.05$); the PROC GLM or ANOVA of SAS 9.2 (SAS Institute, Cary, NC) was used.

Results and discussion

In vitro shoot growth and proliferation

After six weeks on modified MS containing 25 μM 2ip, 60–85% of the nodal segments from each of the four cultivars were sterile and produced shoots (Fig. 1a). On shoot proliferation medium containing modified WPM and 9.12 μM zeatin, stabilized uniform shoot cultures were obtained for all of the four cultivars after four subcultures.

Based on morphological appearance, newly derived shoot cultures on shoot proliferation medium were not stabilized during the first two subcultures. They grew vigorously and 5–20% of them showed some stress symptoms such as red or light green color of stems or leaves. In addition, the unstabilized shoot cultures had thicker stems, bigger leaves, and lower proliferation rates in comparison to the more uniform shoot cultures that were stabilized after four subcultures (Fig. 1b, c).

Adventitious shoot regeneration from leaf explants

To develop efficient regeneration systems for four cultivars, we tested ten PGR treatments. Either the cultivars or the treatments had a significant effect on shoot regeneration (Table 1). Depending on the genotype and on the treatment, shoot regeneration was observed via either pattern I, where the regenerants emerged mostly at the cut edge of explants or wound sites (Fig. 1d), or pattern II, where regenerants appeared on the entire surface of the leaf explants (Fig. 1e). Optimum combination of PGRs for shoot regeneration was genotype-dependent (Table 1).

'Jewel' showed pattern I shoot regeneration, and regeneration occurred only on the medium containing 4.54 μM TDZ and 2.69 μM NAA (Table 1). 'Jubilee' had optimum regeneration on the same medium as 'Jewel' via pattern I; in addition, shoot regeneration occurred on the medium containing zeatin riboside (5.7 or 11.4 μM) via pattern II (Table 1). Both 'Emerald' and 'Biloxi' were amenable to shoot regeneration on different media. On the media containing TDZ (4.54 or 9.08 μM) alone or in combination with NAA, most of the regenerated explants were of pattern I; in contrast, shoot regeneration on the other media was mostly of the pattern II type. 'Emerald' regenerated shoots on seven media, of which five gave over 75% regeneration frequencies. The optimum regeneration medium for 'Emerald' contained either TDZ (4.54 or 9.08 μM), zeatin (18.2 μM), or zeatin riboside (11.4 μM) (Table 1). Shoot regeneration for 'Biloxi' occurred on six PGR treatments, of which four yielded high regeneration frequencies; the optimum regeneration medium contained 11.4 μM zeatin riboside (Table 1).

TDZ-induced buds/shoots elongated very slowly on the same TDZ-containing regeneration medium. On modified WPM containing 9.12 μM zeatin, regenerated shoots from all PGR-treatments for each cultivar continued growth and showed no morphological differences compared to the parent shoot subcultures. Similarly, a two-step regeneration strategy, using TDZ to induce bud/shoot formation followed by using zeatin to promote shoot elongation, has also been reported for northern highbush blueberry cultivars and wild lowbush blueberry (*Vaccinium angustifolium* Ait.) (Song and Sink 2004; Debnath 2009).



Fig. 1 Establishment of shoot cultures and adventitious shoot regeneration of southern highbush blueberry cultivars. **a** Growth of surface-sterilized branch section of ‘Emerald’ on modified MS containing 25 μM 2ip after 6 weeks, **b** Unstabilized shoot cultures of ‘Emerald’ obtained within the first 2–3 subcultures—note the variegated and bleached leaves, **c** Stabilized uniform shoot cultures of

‘Emerald’ obtained by four subcultures. **d** Pattern I shoot regeneration for a leaf explant of ‘Jewel’ cultured on WPM containing 4.54 μM TDZ and 2.69 μM NAA for 12 weeks, **e** Pattern II shoot regeneration for a leaf explant of ‘Emerald’ cultured on WPM containing 18.2 μM Zeatin for 12 weeks, **f** Rooted shoots grown in the greenhouse for 3 months. Bars 1 cm

WPM is the common basal medium used for plant regeneration of *Vaccinium* species. Several PGRs, such as 2ip, zeatin, zeatin riboside, TDZ or NAA, are generally used for shoot regeneration of *Vaccinium* species. TDZ either alone or in combination with NAA or 2ip has enabled efficient shoot regeneration for *Vaccinium* (Hruskoci and Read 1993; Marcotrigiano et al. 1996; Qu et al. 2000; Cao and Hammerschlag 2000; Cao et al. 2002; Song and Sink 2004; Debnath 2005; Meiners et al. 2007). For northern highbush blueberry cultivars, Rowland and Ogden (1992) found that zeatin riboside was more effective than either of the cytokinins, 2ip and zeatin, in promoting shoot regeneration from leaf explants of ‘Sunrise’, whereas ‘Bluecrop’ and ‘Duke’ produced no shoots on any of the media tested. Using either TDZ (1 or 5 μM) or zeatin riboside (20 μM) improved adventitious shoot organogenesis from leaf explants of five cultivars (Cao and Hammerschlag 2000; Cao et al. 2002). Our previous studies showed that optimum PGRs for efficient shoot

regeneration varied among six highbush cultivars, including five northern and one southern cultivar ‘Legacy’. Both TDZ and zeatin allowed efficient shoot regeneration for the ‘Legacy’. TDZ at 1 μM yielded a regeneration frequency of 86.7% and 2.5 shoots per explants. Similarly, zeatin at 18.2 μM enabled a regeneration frequency of 83.3% and 2.2 (Song and Sink 2004). For another southern highbush blueberry cultivar ‘Ozarkblue’, Meiners et al. (2007) reported that 20 μM zeatin was superior to TDZ in promoting shoot regeneration from 46.3% leaf explants and the number of shoots per explant was 7.3. Likewise, in this experiment, our results for four additional cultivars indicated that optimum PGRs for southern highbush blueberries are genotype-dependent. Three PGRs (TDZ, zeatin, and zeatin riboside) were of importance for shoot regeneration. NAA (2.69 μM) in combination with TDZ (4.54 μM) significantly promoted shoot regeneration of cultivars ‘Jewell’ and ‘Jubille’ (Table 1). Lower levels of NAA in combination with TDZ, zeatin, or zeatin riboside

Table 2 Rooting of adventitious shoots of four southern highbush blueberry cultivars

Cultivar	Source of in vitro shoots	Total number of shoots tested	Total number of shoots rooted and survived in the greenhouse	Rooting (%) ^z
'Biloxi'	Regenerated shoots ^y	30	27	90.0 ab
	Micropropagated shoots ^x	30	28	93.3 ab
'Emerald'	Regenerated shoots ^y	30	24	80.0 b
	Micropropagated shoots ^x	30	26	86.7 ab
'Jewel'	Regenerated shoots ^y	30	28	93.3 ab
	Micropropagated shoots ^x	30	27	90.0 ab
'Jubilee'	Regenerated shoots ^y	30	30	100.0 a
	Micropropagated shoots ^x	30	29	96.7 a

Different letters in the same column denote significant differences at the 5% level by Duncan's test. $n = 24$

^x The shoots were excised from 8 week-old, stem-derived shoot cultures maintained on modified WPM containing 9.12 μM zeatin

^y The shoots were derived from leaf explants on optimum regeneration medium and were subsequently cultured for 8 weeks on modified WPM containing 9.12 μM zeatin

^z Rooting (%) = Total number of shoots rooted and survived in the greenhouse/Total number of shoots tested × 100

might further improve shoot regeneration but they were not tested in this study. In compared to the regeneration system for 'Legacy' which allowed a routine transformation using *Agrobacterium tumefaciens* (Song and Sink 2004; Song et al. 2008), the optimized regeneration systems for the four cultivars used in this study have potential use for transformation studies.

Effect of leaf explants on shoot regeneration

Newly derived shoot cultures from softwood cuttings were not uniform in appearance during the first three subcultures due probably to the interaction between endogenous hormones in the shoots and the PGR(s) (2ip or zeatin) added in shoot induction medium. After four subcultures, the impact of endogenous hormones was reduced and shoot cultures with a uniform physiological state were obtained. For all four cultivars, leaf explants from stabilized uniform shoot cultures resulted in significantly higher regeneration frequencies than those from unstabilized shoot cultures (Table 1). These results were consistent with our previous observations on northern highbush blueberry cultivars (unpublished data), indicating that the physiology of leaf explants is important for adventitious shoot regeneration. The specific physiological state of the initial leaf explants may be required to initiate more competent cells for regeneration.

Rooting

In previous reports, ex vitro rooting of in vitro shoots has been very successful for blueberry cultivars (Lyrene 1981; Song and Sink 2004; Meiners et al. 2007). Similar results were obtained in this study. Regenerated shoots, 80–100%

for each cultivar, rooted in 8 weeks after they were transplanted into soil. All plants transferred to the greenhouse survived. For each cultivar, the rooting percentages of regenerated shoots showed no statistical difference with those of micropropagated shoots (Table 2). Plant derived from regeneration, micropropagation and softwood cuttings showed no apparent difference in morphology after 3 months growing in the greenhouse (Fig. 1f).

Conclusions

Efficient regeneration systems with high regeneration frequencies (53.3–88.9%) and numbers of shoots per explant (3.16–13.2) were developed for four southern highbush blueberry cultivars. The optimum regeneration PGRs for adventitious shoot regeneration of southern highbush blueberries was genotype dependent. Leaf explants from stabilized shoot cultures were preferable to those from unstabilized shoot cultures for efficient regeneration. The regeneration systems described have potential use in genetic transformation of southern highbush blueberry cultivars.

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